Interferon Regulatory Factor 6 Promotes Differentiation of the Periderm by Activating Expression of Grainyhead-Like 3

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IFN regulatory factor 6 (IRF6) is a transcription factor that, in mammals, is required for the differentiation of skin, breast epithelium, and oral epithelium. However, the transcriptional targets that mediate these effects are currently unknown. In zebrafish and frog embryos, Irf6 is necessary for differentiation of the embryonic superficial epithelium, or periderm. Here we use microarrays to identify genes that are expressed in the zebrafish periderm and whose expression is inhibited by a dominant-negative variant of Irf6 (dnIrf6). These methods identify Grainyhead-like 3 (*Grhl3*), an ancient regulator of the epidermal permeability barrier, as acting downstream of Irf6. In human keratinocytes, IRF6 binds conserved elements near the *GRHL3* promoter. We show that one of these elements has enhancer activity in human keratinocytes and zebrafish periderm, suggesting that Irf6 directly stimulates *Grhl3* expression in these tissues. Simultaneous inhibition of *grhl1* and *grhl3* disrupts periderm differentiation in zebrafish, and, intriguingly, forced *grhl3* expression restores periderm markers in both zebrafish injected with *dnIrf6* and frog embryos depleted of *Irf6*. Finally, in *Irf6*-deficient mouse embryos, *Grhl3* expression in the periderm and oral epithelium is virtually absent. These results indicate that Grhl3 is a key effector of Irf6 in periderm differentiation.

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INTRODUCTION

IFN regulatory factor 6 (IRF6) drives the differentiation of epithelia with barrier function via unknown mechanisms. In mice deficient for *Irf6*, epidermal keratinocytes fail to differentiate terminally, resulting in the absence of an epidermal permeability barrier (Ingraham *et al.*, 2006; Richardson *et al.*, 2006; Biggs *et al.*, 2012). Moreover, the superficial layer of the oral epithelium, the oral periderm, is missing in *Irf6* mutants, resulting in adhesion between the palatal shelves and the tongue (Richardson *et al.*, 2009). Mutations in *IRF6* cause two syndromic forms of cleft lip and palate (CL/P): the Van der Woude and the Popliteal Pterygium syndromes (Kondo *et al.*, 2002). They are also associated with nonsyndromic CL/P (Zucchero *et al.*, 2004; Koillinen *et al.*, 2005; Srichomthong *et al.*, 2005; Pegelow *et al.*, 2008; Desmyter *et al.*, 2010). In patients with such mutations, abnormal differentiation of the epidermis or oral periderm may contribute to CL/P pathogenesis. In addition, IRF6 appears to be necessary for normal wound healing (Jones *et al.*, 2010), it promotes differentiation of the breast epithelium (Bailey *et al.*, 2008), and it functions as a tumor suppressor in squamous cell carcinoma (Botti *et al.*, 2011). A recent study identified hundreds of genomic loci that are bound by IRF6 in human keratinocytes (Botti *et al.*, 2011), but it is unclear which of these apparent transcriptional targets effect epithelial differentiation.

The aim of this study was to characterize the position of Irf6 within the gene regulatory network that controls differentiation of the periderm in zebrafish, as many aspects of mammalian skin biology are conserved in fish (Li *et al.*, 2011). The periderm is a simple squamous epithelium that is the most superficial layer of an embryo and forms the first

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Abbreviations: cDNA, complementary DNA; CL/P, cleft lip and palate; dnIrf6, dominant-negative variant of Irf6; Grhl3*, Grainyhead-like 3; GFP, green fluorescent protein; h.p.f., hours post fertilization; IRF6, IFN regulatory factor 6; MO, morpholino oligonucleotide; mRNA, messenger RNA*

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permeability barrier to dehydration in mouse embryos (M'Boneko and Merker, 1988). In both fish and amphibian embryos, the periderm forms in early gastrula stage and is necessary for structural integrity of the embryo during this period (Fukazawa *et al.*, 2010). We previously showed that the periderm is disrupted in both zebrafish embryos injected with RNA encoding a dominant-negative variant Irf6 (dnIrf6) and in frog embryos depleted of maternal Irf6: the expression of various periderm markers is lost, and the periderm itself ruptures (Sabel *et al.*, 2009). Here, through microarray analysis, *in vivo* reporter studies, and epistasis experiments, we identify the gene encoding Grainyhead-like 3 (*Grhl3*), an ancient mediator of epithelial integrity, as a direct effector of Irf6 in periderm differentiation.

RESULTS

Potential regulators of periderm differentiation

We assessed the gene expression profile of isolated zebrafish periderm cells using the *Tg(krt4:gfp)* transgenic zebrafish line, in which green fluorescent protein (GFP) is robustly expressed in simple epithelia of the embryo (Gong *et al.*, 2002). Through cell sorting and microarray analyses, we identified a subset of genes expressed in GFP^+ cells at levels at least threefold higher than in GFP^- cells (Figure 1a and b). This ''periderm-enriched profile'' (Supplementary Table S1 online) is significantly enriched for genes with ontology terms typical of those present in other epithelia (See Materials and Methods). The 70 transcription factor–encoding genes represented in this profile (Supplementary Table S1 online) potentially participate in the periderm gene regulatory network, either upstream or downstream of Irf6.

To learn where Irf6 fits into this network, we created a profile of genes whose expression is altered in the presence of dnIrf6. We did not use antisense morpholino oligonucleotides (MO), the standard tools for reducing gene expression in zebrafish embryos, because they are commonly ineffective against maternally encoded transcripts (Draper *et al.*, 2001), and this proved to be the case for *Irf6* (Supplementary Figure S1 online). Instead, we modified the dnIrf6 generated for our earlier study (Sabel *et al.*, 2009) by fusing the Irf6 DNA-binding domain to the Engrailed repressor domain. We injected embryos with an RNA encoding either dnIrf6 or β-galactosidase (i.e., *lacZ* RNA), harvested RNA at 6 hours post fertilization (h.p.f.; such embryos rupture by 7 h.p.f.), and probed microarrays as described above. We identified a subset of genes that are expressed at significantly lower levels in *dnIrf6*-injected embryos than in *lacZ*-injected embryos and refer to this as the ''dnIrf6-inhibited profile'' (Figure 1c and d, Supplementary Table S2 online); it contains many genes that encode adhesion molecules, consistent with rupture of the periderm in dnIrf6-injected embryos.

Of the genes present in both the periderm-enriched and dnIrf6-inhibited profiles (Figure 1e; Supplementary Table S3 online), 10 are classified by the gene ontology term ''transcription factor activity'' and therefore may participate in the periderm gene regulatory network downstream of Irf6 (Table 1). Among these, *grhl1* and *klf2b* are of special interest because homologs of these genes, *Grhl3* and *Klf4*, respectively, are required to generate the epidermal permeability barrier in mice (Segre *et al.*, 1999; Ting *et al.*, 2005). However, probes for the zebrafish orthologs of neither *Grhl3* nor *Klf4* are present in the microarray we deployed. Therefore, we assessed the expression of these genes, and that of several others common to the two profiles, by *in situ* hybridization. We found that all are indeed downregulated in *dnIrf6*-injected embryos versus control-injected embryos (Figure 1f–w), and that *epcam*, which is present only in the former profile, is indeed expressed in dnIrf6-injected embryos (Figure 1x and y). Moreover, we found that coinjecting an RNA encoding full-length Irf6 partially reversed the effects of *dnIrf6* (Figure 1z), supporting the idea that those effects result from interference with Irf6 and/or closely related proteins. Genes in the periderm-enriched profile whose expression was significantly *elevated* in the presence of dnIrf6 are candidates for repression by Irf6, although most are likely to be affected indirectly because dnIrf6 is a constitutive repressor. Interestingly, *p63*, which regulates epidermal differentiation in mice and zebrafish and directly regulates Irf6 expression, ranks among these genes (Bakkers *et al.*, 2002; Lee and Kimelman, 2002; Moretti *et al.*, 2010; Thomason *et al.*, 2010). In summary, profiling experiments revealed several genes that are likely to be members of the periderm gene regulatory network that act downstream of Irf6.

Irf6 activates epithelial enhancers adjacent to *Grhl3*

We next sought to test the possibility that Irf6 directly activates *grhl3* expression in the periderm. In a recent chromatin immunoprecipitation experiment conducted in human keratinocytes, IRF6 was found to be bound at two locations near the first exon of *GRHL3*—about 2 kb upstream and about 3 kb downstream (intronic; Figure 2a; Botti *et al.*, 2011). Both peaks are within chromatin elements that are conserved among mammals and are sites of DNAse hypersensitivity in human keratinocytes (ENCODE project). Thus, these elements may have *cis*-regulatory function. In addition, microarray analysis revealed that *GRHL3* expression is reduced in cells depleted of *IRF6*, suggesting that IRF6 directly activates *GRHL3* expression in keratinocytes (Botti *et al.*, 2011). To test this possibility, we amplified the conserved elements containing these peaks and cloned them into a firefly luciferase reporter vector containing a minimal promoter (Figure 2a). In a human keratinocyte cell line transfected with the construct containing the intron-1 element, but not in one transfected with the construct containing the upstream element, luciferase levels were significantly higher than in a line transfected with a control construct (Figure 2b). Although neither IRF6-bound element is detectably conserved in zebrafish, the functions of mammalian enhancer elements without detectable counterparts in zebrafish are nonetheless often conserved in zebrafish embryos (Fisher *et al.*, 2006; McGaughey *et al.*, 2008, 2009). We thus cloned the elements into a reporter vector that contains a minimal promoter and the gene encoding GFP (Figure 2a; vector described in Fisher *et al.* (2006)) and injected these constructs into wild-type zebrafish embryos. About 20% of the embryos injected with

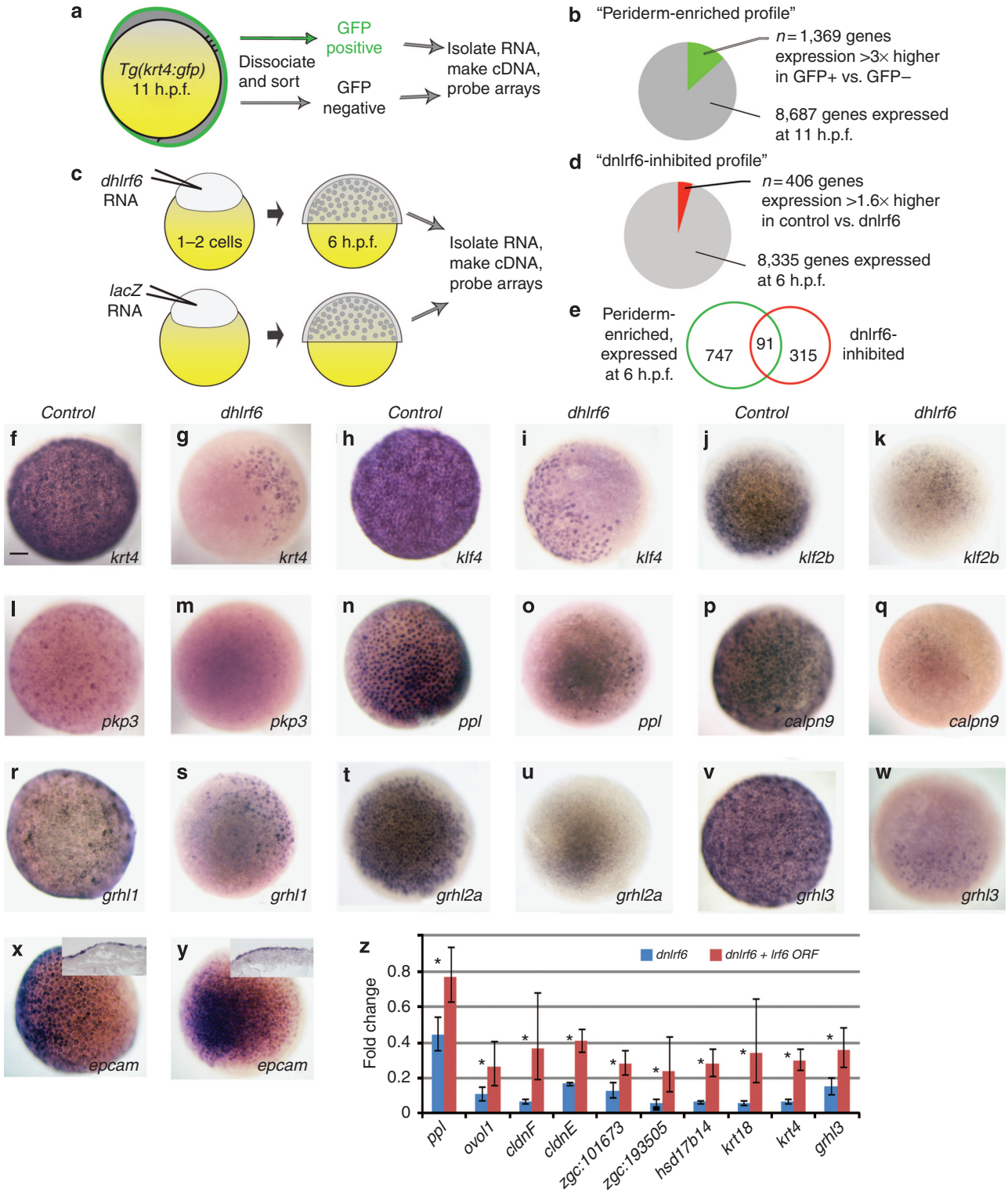


Figure 1. Microarray analysis of zebrafish periderm. (**a**, **c**) Schematic representation of methods used to create the periderm-enriched (**a**) and the dominant-negative variant of IFN regulatory factor 6 (dnIrf6)-inhibited (**c**) profile. (**b**, **d**) Pie charts showing genes whose expression is (**b**) 3 × higher in FACS-sorted green fluorescent protein (GFP)-positive versus GFP-negative cells (**c**) or significantly reduced (>1.7-fold) in *dnIrf6*-injected compared with *lacZ*-injected embryos (**d**). (**e**) Venn diagram portraying the overlap of sets in **b** and **d**. (**f**–**y**) Animal-pole view of midgastrula-stage embryos, uninjected or injected with *dnIrf6*, and processed to reveal expression of the indicated gene. All are expressed in periderm and all, except **y** (*epcam*), are strongly reduced in dnIrf6-injected embryos. Bar = 100 μM (**f**). (**z**) Quantitative reverse transcription PCR for genes whose expression is reduced by *dnIrf6* and significantly elevated (with respect to dnIrf6 alone) by coinjection with *Irf6* ($P<0.05$) (asterisks). cDNA, complementary DNA; h.p.f., hours post fertilization.

Table 1. Genes encoding transcription factors in both the periderm-enriched and dnIrf6-inhbited profiles

Gene ID	Gene name
ENSDART00000082425	GATA-binding protein 2a
ENSDART00000021159	LIM homeobox 1b
ENSDART00000080693	LIM homeobox 5
ENSDART00000060861	Forkhead box P2
ENSDART00000025153	GATA-binding protein 3
ENSDART00000052521	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1
ENSDART00000052570	Paired-like homeodomain transcription factor 2
ENSDART00000017456	Ventral expressed homeobox
ENSDART00000087454	Grainyhead-like 1
ENSDART00000059188	Klf2b, Kruppel-like factor 2b

hGRHL3_peak2:gfp exhibited GFP expression in contiguous patches of periderm cells (recognizable because of their characteristic polygonal morphology), starting at high stage and continuing to at least 72 h.p.f. (shown on the yolk at 48 h.p.f., Figure 2c; numbers of injected embryos for reporter experiments are presented in Materials and Methods). Embryos injected with *hGRHL3_peak1:gfp* or the vector lacking either element did not exhibit these patches. These studies support the idea that Irf6 directly activates *grhl3* expression in both human keratinocytes and zebrafish periderm.

As an alternative approach for identifying Irf6-dependent enhancers, we searched mammalian and zebrafish genomes for the presence of conserved, noncoding elements that possess the consensus Irf6 response element (Little *et al.*, 2009). Although we did not detect any that are conserved between mammals and zebrafish, we found one (within intron 15 of *GRHL3*) that is conserved among mammals (Figure 2d). Although IRF6 binding of this site was not reported in keratinocytes (Botti *et al.*, 2011), it could

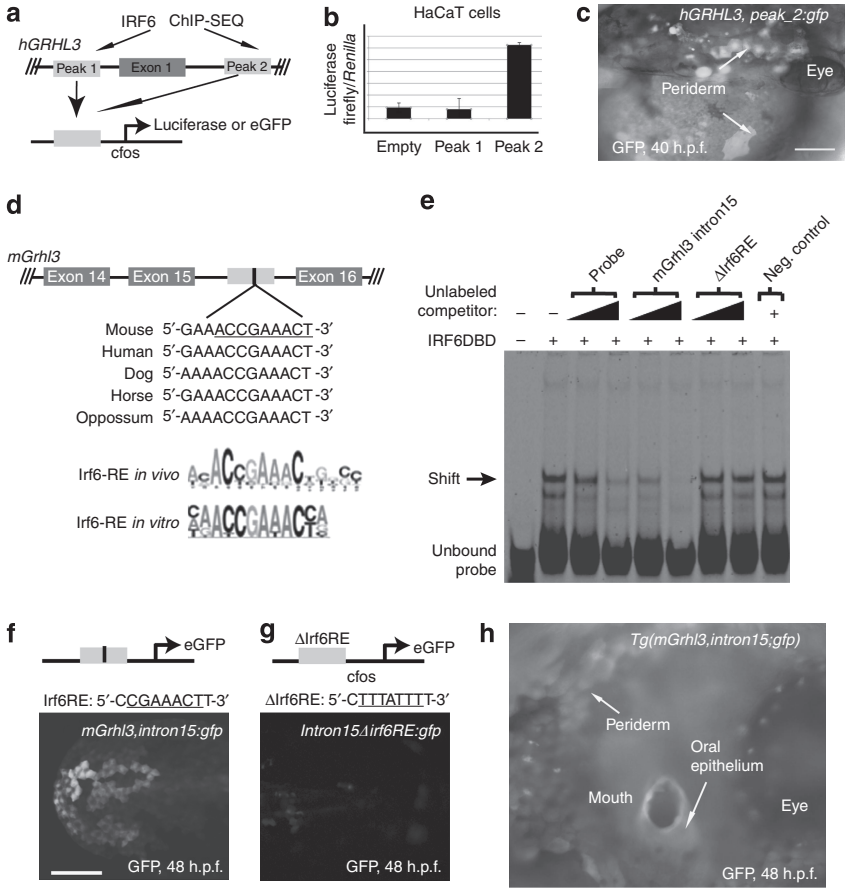
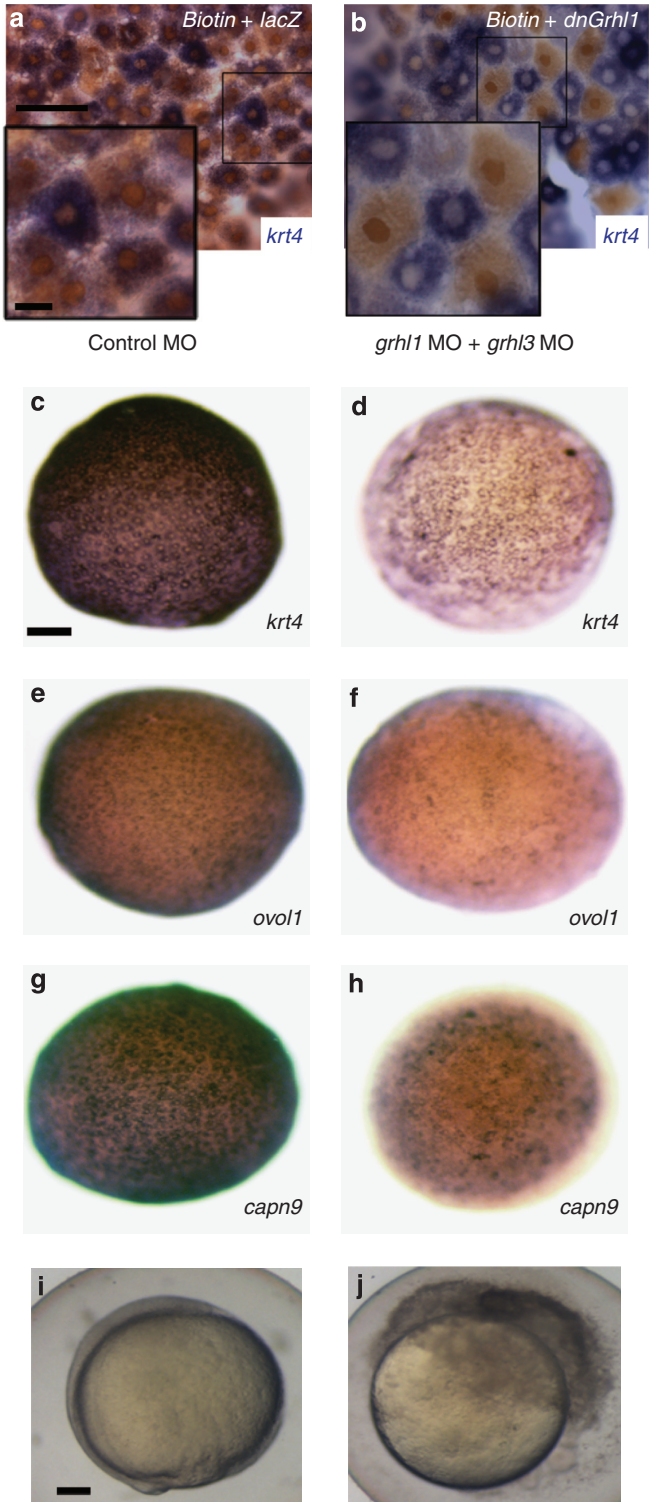


Figure 2. Enhancer activity of DNA elements near *Grainyhead-like 3* (*GRHL3*). **(a)** Positions of Irf6 binding peaks in human keratinocytes from a ChIP-SEQ experiment (Botti *et al.*, 2011), and construction of luciferase and green fluorescent protein (GFP) reporter vectors with these peaks. (**b**) Results of luciferase reporter assay. (**c**, **f**–**h**) Transient (**c**, **f**, **g**) and stable (**h**) transgenic zebrafish embryos of the indicated constructs with GFP expression in the periderm. (**d**) Schematic representation of a conserved element within *GRHL3* intron-15 (gray box), which contains a conserved Irf6 response element (IRF6-RE), underlined. Position-weighted matrix for IRF6-RE defined *in vitro* (Little *et al.*, 2009) and *in vivo* (Botti *et al.*, 2011). (**e**) Electromobility shift assay with human IRF6DBD and a labeled probe that matches 25 base pairs of the *hGrhl3 intron 15* that encompass the putative Irf6RE. Bars = (**c**) 100 μm and (**f**) 200 μm. eGFP, enhanced green fluorescent protein; h.p.f., hours post fertilization.

potentially occur in other cell types including those of the periderm. Indeed, we found that an oligonucleotide that matches the *GRHL3* intron sequence encompassing the apparent Irf6 binding site competes with a control probe for the binding of Irf6DBD *in vitro*, and that a version of the oligo in which this site has been altered lacks this ability (Figure 2e). To test the potential enhancer activity of the conserved element within *GRHL3* intron-15, we amplified it (972 bp) from the murine genome and cloned it into the GFP reporter vector (creating *mGrhl3intron15:gfp*). In both transient and stable transgenic embryos harboring this construct, GFP is expressed in the periderm (Figure 2f and h). In addition, we generated a construct in which six bases within the Irf6RE are mutated (as in the *in vitro* binding experiment described above), creating *intron15ΔIrf6RE:gfp* (Figure 2g). In embryos injected with this construct, GFP expression was rarely detected in periderm cells and was completely extinguished by 48 h.p.f. (Figure 2g). In summary, a conserved element within intron-15 of mammalian *Grhl3* has periderm enhancer activity that depends on the presence of a consensus Irf6 binding site.



Inhibition of *grhl1* and *grhl3* disrupts periderm development

Three members of the Grhl family are expressed in the zebrafish periderm (Janicke *et al.*, 2010). Because Grhl family members share a core DNA consensus binding site (Ting *et al.*, 2005; Wilanowski *et al.*, 2008; Rifat *et al.*, 2010; Boglev *et al.*, 2011), we reasoned that overexpressing the DNA-binding domain (DBD) of any is likely to displace them all from DNA. We thus injected zebrafish embryos with an RNA encoding the *Xenopus* Xgrhl1 DBD (*dnXgrhl1*). This resulted in stalled epiboly and embryonic rupture, much as in *dnIrf6*-injected embryos (not shown, 29 of 30 injected embryos; Tao *et al.*, 2005). We found that mosaic injection of *dnXgrhl1* blocked *krt4* expression in a cell autonomous manner (Figure 3a and b). To determine which family members are essential for development of the zebrafish periderm, we injected MOs known to target the *grhl1* start codon (Janicke *et al.*, 2010) or to block the splicing of *grhl3* (confirmed in Supplementary Figure S2 online). Epiboly was not affected when either was injected alone, but when both were injected simultaneously the expression of *krt4*, *capn9*, and *ovol1b* was significantly reduced, epiboly was delayed, and the embryos ruptured analysis at the animal pole (Figure 3c–j). Reverse transcriptase PCR (i.e., qRT-PCR) analysis confirmed that the combination of MOs targeting *grhl1* and *grhl3* reduced expression of *krt4* (Supplementary Figure S2 online). Thus, Grhl1 and Grhl3 function redundantly to promote periderm differentiation in zebrafish.

Figure 3. Knockdown of *grhl* family members results in loss of periderm markers. (**a**, **b**) Animal-pole view of 8 hours post fertilization (h.p.f.) embryos injected mosaically with (**a**) biotinylated dextran mixed with *lacZ* or (**b**) *dnXgrhl1* messenger RNA. Embryos were fixed at shield stage and processed to reveal *krt4* expression (purple) and biotin (brown). Periderm cells inheriting *lacZ* (brown nuclei) express *krt4* variably, like periderm cells in uninjected embryos, whereas those inheriting *dnXGrhl1* (brown nuclei) lack *krt4*. (**c**–**j**) Embryos injected with control morpholino oligonucleotide (MO) (**c**, **e**, **g**, **i**), or *grhl1* and *grhl3* MOs (**d**, **f**, **h**, **j**), fixed at 8 h.p.f., and processed to reveal expression of the indicated gene, or allowed to develop until 10 h.p.f. (**i**–**j**), at which time control MO-injected embryos have normal morphology (**i**), and *grhl1/grhl3* MO-injected embryos are ruptured (**j**). Bars = (**a**) 50 μm; (**a** inset) 10 μm; (**c**) 100 μm; (**i**) 100 μm.

Forced expression of *grhl3* partially restores epiboly in dnIrf6-injected embryos

During epiboly, blastomeres in control embryos migrate toward the vegetal pole, producing doming of the yolk toward the animal pole (Figure 4g and h). In the large majority of embyros injected with *dnIrf6* messenger RNA (mRNA) and subsequently injected with a control mRNA (i.e., *lacZ*), these movements do not appear to occur (Figure 4h), and virtually all have ruptured through the animal pole by the time control embryos reach 6.5 h.p.f. (Figure 4e; 94%, $n=114$ injected embryos). By contrast, in the majority of *dnIrf6*-injected embryos that are subsequently injected with *grhl3* mRNA, blastomeres move toward the vegetal pole, the yolk undergoes doming, and embryos are still alive at 6.5 h.p.f. (Figure 4f and i; 70%, $n=119$). Moreover, they express the periderm markers *claudinE*, *capn9*, and *ovol1* at higher levels, as demonstrated by qRT-PCR analysis (Figure 4j) and confirmed by *in situ* hybridization for *capn9* (Figure 4k–m). Most such embryos, nonetheless, ultimately rupture by the time control embryos reach 7.5 h.p.f. (81%, $n=119$). Thus, *grhl3* mRNA improved survival and morphogenesis during epiboly of dnIrf6-injected embryos.

We also tested the ability of Grhl3 to reverse the effects of MO-mediated knockdown of the *irf6* mRNA in frog embryos. Using the oocyte-transfer method to inhibit maternal *irf6*, we confirmed the earlier report (Sabel *et al.*, 2009) of a strong reduction of the superficial epithelium marker *Xk81a1* and upregulation of the deep cell marker *sox11* (Figure 4n). In embryos that were additionally injected with the *Xenopus grhl3* mRNA, *xk81a1* levels were significantly closer to those in control embryos (Figure 4n). Thus, Irf6-mediated activation of *grhl3* expression appears to be a critical component in the periderm gene regulatory network in fish and frogs.

Expression of *Grhl3* in murine *Irf6*-deficient embryos

We harvested epidermis from mouse embryos that expressed wild-type and/or a null allele of *Irf6* (i.e., homozygous control, heterozgous mutant, or homozygous mutant) at E14.5 and E17.5, and measured *Grhl3* levels by qRT-PCR analysis. In homozygous mutants, *Grhl3* was reduced at E14.5 but elevated at E17.5 with respect to the levels in siblings (wild-type or heterozygous mutant; Figure 5a). The elevation at the later time point is consistent with the observation that epidermal stratification is abnormal (the basal and spinous layers are expanded and exhibit inappropriate gene expression, and the granulosum and cornified layers are absent; Ingraham *et al.*, 2006; Richardson *et al.*, 2006). To determine the reason for the reduction in *Grhl3* expression at E14.5, we evaluated anti-Grhl3 immunoreactivity in coronal head sections from homozgyous *Irf6* mutants and their siblings at E14.5. In the sibling embryos, nuclear Grhl3 immunoreactivity was detected in all layers of the epidermis, with expression highest in periderm cell nuclei (Figure 5b). In *Irf6* mutants, Grhl3 immunoreactivity was generally lower and non-nuclear, and the characteristic periderm expression was absent (Figure 5c). Similar changes in the gene expression pattern were observed in the oral

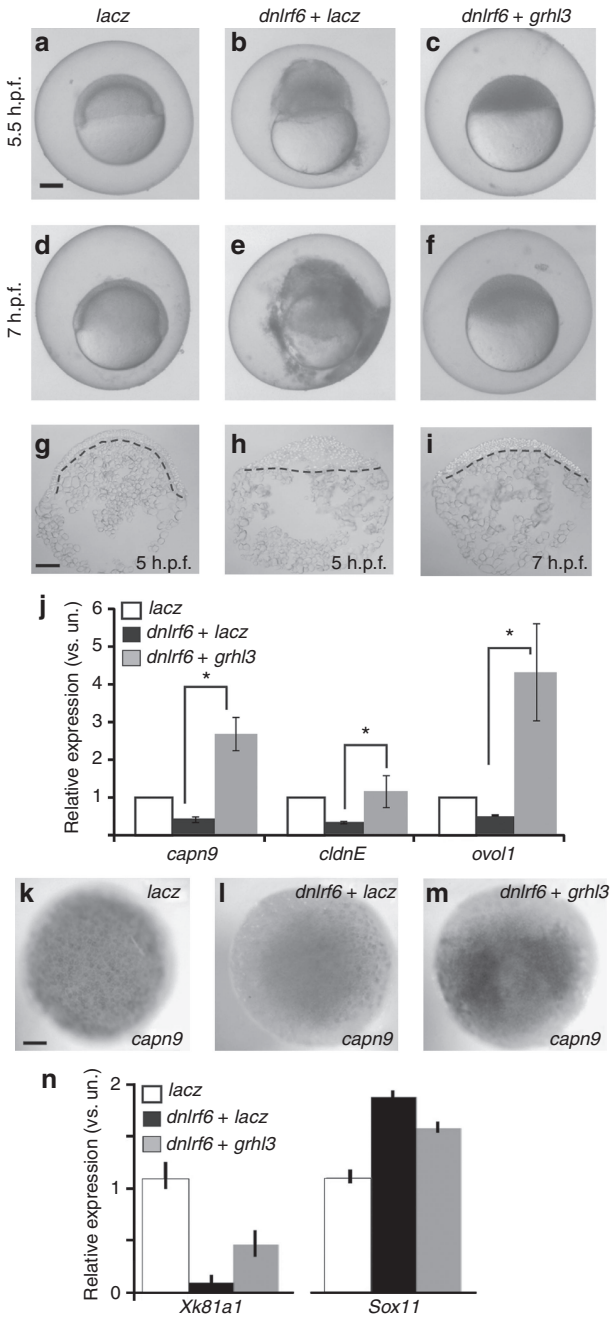


Figure 4. Injection of *Grainyhead-like 3* (*grhl3*) restores periderm markers in *dominant-negative variant of IFN regulatory factor 6* (*dnIrf6*)-injected embryos. (**a**–**i**) Images of live (**a**–**f**) and 4′-6-diamidino-2-phenylindole-stained sections of (**g**–**i**) embryos injected with the indicated RNA. Insets, magnified images of the boxed area. (**g**–**i**) The doming of the yolk apparent in lacz-injected embyros (**g**) is lost in *dnIrf6*-injected embryos (**h**) and partially restored in *dnIrf6*- and *grhl3*-injected embryos (**i**). (**j**) Quantitative reverse transcription PCR (qRT-PCR) analysis of messenger RNA (mRNA) levels. *capn9*, $P=0.03$, *cldnE*, $P=0.003$, *ovol1*, $P=0.048$. (**k**–**m**) 5-Hours post fertilization (h.p.f.) embryos processed for *capn9* expression. (**n**) qRT-PCR analysis of levels of the periderm marker *xk81a1*, and the deep blastomere marker *sox11*, in *Xenopus* embryos derived from oocytes injected with control morpholino oligonucleotide (MO) or *irf6* MO and injected as zygotes with the *XGrhl3* mRNA, as indicated. By analysis of variance, *xk81a1*, $P<0.0002$, *sox11*, $P<0.03$. Error bars, standard deviation. Bars = (**a**) 200 μm; (**g**) 100 μm; (**k**) 100 μm.

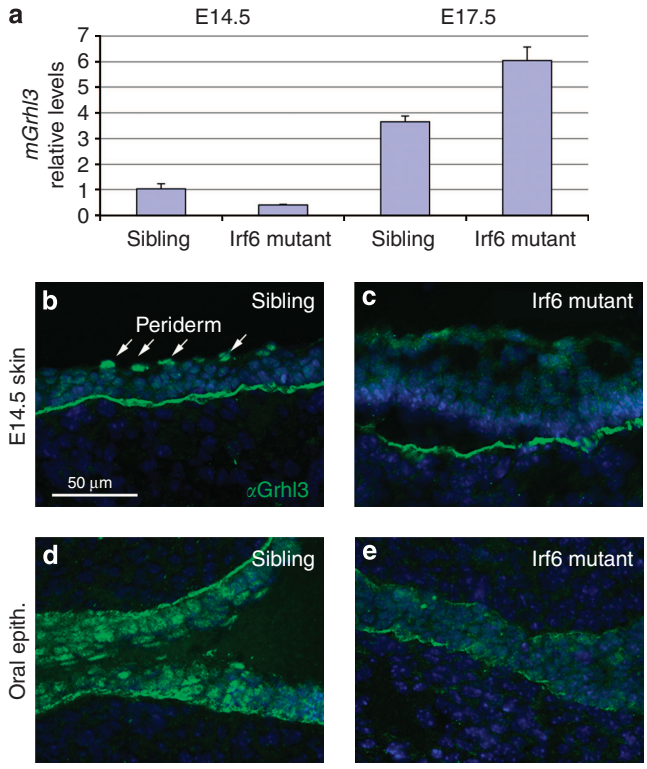


Figure 5. Expression of Grhl3 in *Irf6*-deficient mice. (**a**) Quantitative reverse transcription PCR analysis of *Grhl3* levels in epidermis harvested at the indicated stage: E14.5, two replicates; E17.5, three replicates; $P<0.03$. (**b**–**e**) Anti-Grhl3 immunofluorescence on the indicated tissue at the indicated stages. In the epidermis of sibling control embryos (**b**), Grhl3 immunoreactivity (IR) is prominent in all epidermal and peridermal nuclei. In mutant embryos (**c**), Grhl3 IR is diffuse, non-nuclear, and weak or undetectable in the periderm. In the oral epithelium (epith.) of control siblings (**d**), Grhl3 IR is strong in all nuclei and strongest in the oral periderm. In mutant embryos (**e**), Grhl3 IR is highly reduced. (**b**) Bar = 50 μm.

epithelium (Figure 5d and e). Together, these findings suggest that Irf6 directly activates *Grhl3* expression in the periderm and oral epithelium in mice, and that in keratinocytes Irf6 contributes to the activation of *Grhl3* expression but is not essential for it.

Finally, we generated mutants doubly heterozygous for Irf6 and Grhl3. In newborn $Irf6^{gt/+}$; $Grhl3^{del/+}$ pups, we detected neither gross morphological defects in the face, ears, limbs, or oral cavity nor obvious histological defects in skin or oral epithelium ($n=6$; not shown). Thus, if Irf6 and Grhl3 function in the same pathway during formation of the permeability barrier, the dosage of neither gene is limiting in double heterozygotes.

DISCUSSION

Irf6 and the periderm gene regulatory network

Here we have conducted profiling experiments that have shed light on the role of Irf6 in zebrafish periderm development. We used conservative criteria in identifying genes in the periderm-enriched and dnIrf6-inhibited profiles, and probes for some of the genes that are important for epithelial development, including *grhl3* itself, were not represented on the microarray we deployed. Nonetheless, the periderm-enriched profile includes at least 70 genes encoding transcription factors, all of which are candidate participants in the periderm transcriptional gene regulatory network. Those that are also present in the *dnIrf6*-inhibited profile include *grhl1*, *tfap2c*, *klf2b*, and *c/ebp beta*. Each of these genes or a homolog has previously been implicated in epithelial development. Notably, many of the regulatory genes in the periderm profile are not in the *dnIrf6*-sensitive profile, and these could play roles upstream of, or parallel to, Irf6 during epithelial development. Candidates for regulatory proteins acting upstream of Irf6 include TFAP2A (Rahimov *et al.*, 2008), aPKC (Chalmers *et al.*, 2003), Ikk1 (Fukazawa *et al.*, 2010), and p63 (Moretti *et al.*, 2010; Thomason *et al.*, 2010). Thus, our work has helped to place Irf6 within the gene regulatory network that governs differentiation of the zebrafish periderm, a tissue that serves as a model for other simple squamous epithelia, including mammalian oral periderm.

Grhl3 appears to be a direct effector of Irf6 in the periderm

Our results suggest that Irf6 directly regulates *grhl3* expression and that in some tissues it may do so in conjunction with other Irf family members. Specifically, we show that *Grhl3* expression is strongly downregulated in the periderm of zebrafish embryos injected with dnIrf6, consistent with previous findings from *Xenopus* embryos derived from oocytes injected with *irf6* MO (Sabel *et al.*, 2009), *Irf6* mutant mouse embryos (Ingraham *et al.*, 2006; Richardson *et al.*, 2006, 2009), and human keratinocytes transfected with an small hairpin RNA targeting human IRF6 (Botti *et al.*, 2011). Moreover, we have identified two conserved noncoding elements close to *GRHL3*—one known to be bound by IRF6 in keratinocytes, and another strongly conserved Irf6 consensus binding site—both of which have zebrafish periderm enhancer activity. We have also found that *Grhl3* expression is not completely dependent on Irf6 in all tissues—for instance, in mouse keratinoctyes; it is possible that in such contexts Irf6 homologs cooperate with Irf6 to regulate target gene expression. We note that the periderm-enriched profile includes two *irf6* homologs (namely, *irf4a* and *irf11*). The presence of these genes, together with maternally encoded *irf6*, may explain why dnIrf6 effectively reduces *grhl3* expression upon injection into zebrafish eggs, whereas the irf6 MO does not (our unpublished finding). We also note that a chromatin precipitation with next generation sequencing (ChIP/SEQ) experiment found that IRF4 binds intron-15 of *GRHL3* in a lymphoblast cell line (ENCODE project, Rick Myers laboratory, HudsonAlpha Institute). In summary, the available genetic and biochemical evidence indicates that Irf6, possibly in conjunction with its homologs, directly activates *grhl3* expression in epithelial structures.

Clinical and evolutionary implications

Because Grhl3 appears to act in concert with Irf6 in the oral periderm gene regulatory network, mutations in *GRHL3*, and in genes encoding other members of this network, are candidates that contribute risk for CL/P. Although mutations

in *IRF6* have been estimated to confer 12% of the inherited risk for nonsyndromic CL/P (Zucchero *et al.*, 2004), the majority of such a risk has not been ascribed to any gene (Yuan *et al.*, 2011). In mice, Irf6 is necessary for differentiation of the oral epithelium, particularly that of the oral periderm (Richardson *et al.*, 2009). *Grhl3* is expressed in the zebrafish oral epithelium (our unpublished findings) and in mouse embryos (Auden *et al.*, 2006). Furthermore, as recently reported, mouse *Grhl2* mutants exhibit orofacial clefts (Pyrgaki *et al.*, 2011). Although our macroscopic analyses of $Irf6^{gt/+}$;$Grhl3^{del/+}$ double-heterozygous mice at birth did not detect gross anomalies in skin development, it will be important to assess embryos for the presence of oral adhesions. Importantly, the absence of a phenotype in double heterozygotes is not strong evidence against two genes acting in the same pathway. Indeed, the related defects in barrier function in *Grhl3* and *Irf6* mutants suggest that the two genes promote skin differentiation through the same pathway (Ting *et al.*, 2005; Ingraham *et al.*, 2006; Richardson *et al.*, 2006). In addition, the ability of *Grhl3* to rescue the expression of periderm markers in dnIrf6-injected embryos, as shown here, is strong evidence that they act in the pathway that promotes periderm differentiation. The data presented here motivate an assessment of overtransmission of specific alleles of *GRHL3* (or other *GRHL* family members) in patients with nonsyndromic CL/P, and of mutations in *GRHL3* in patients with syndromic forms of CL/P. In this context, it is intriguing that three linkage studies have detected a risk locus at 1p36, where *GRHL3* resides (Prescott *et al.*, 2000; Martinelli *et al.*, 2001; Moreno *et al.*, 2004), and Van der Woude Syndrome 2 (VWS2), which includes orofacial clefting, maps to an interval that contains *GRHL3* (Koillinen *et al.*, 2001).

Finally, our data lead us to speculate how the role of IRF6 in epithelial development evolved. The IRFs are ancient, having arisen alongside multicellularity in animals (Nehyba *et al.*, 2009); the GRHL family is also ancient, having arisen before the split of arthropods and chordates (Ting *et al.*, 2005). Most members of the IRF family regulate the expression of genes that encode IFNs and proteins that promote the differentiation of dendritic cells (Gabriele and Ozato, 2007). Activation by IRF6 of *GRHL3*, which encodes a regulator of epithelial integrity, does not appear to fit this pattern, suggesting this regulatory interaction is an evolutionary novelty. However, given that the most important aspect of innate immunity is the epidermal permeability barrier, a unifying theme of IRF targets is their involvement in innate immunity. It is plausible that activation by IRF6 of *GRHL3* reflects an ancient interaction that evolved as one feature of the innate immune response.

MATERIALS AND METHODS

Dissociation of zebrafish embryos, FACS, and RNA preparation

For production of the periderm-enriched profile, about 200 heterozygous transgenic *tg(krt4:gfp)* (Gong *et al.*, 2002) embryos were reared to 11 h.p.f., pestle homogenized, and dissociated with phosphate-buffered saline containing 0.25% trypsin and 1 mM EDTA (Gibco, # 25200, Invitrogen, Carlsbad, CA) for 30 minutes at 33 °C. Cells were resuspended in phosphate-buffered saline plus 3% fetal bovine serum and sorted on a FACS DiVa (Becton Dickinson, Franklin Lakes, NJ), directly into a buffer containing guanidinium thiocyanate for subsequent RNA isolation using the RNEasy Plus Mini Kit (i.e., Buffer RLT, QIAGEN, Valencia, CA). RNA was subjected to two rounds of amplification using the Message Amp II aRNA Amplification Kit (Invitrogen). For production of the dnIrf6-inhibited profile, embryos were injected with *dnIrf6* or the *lacZ* mRNA, raised to 6 h.p.f., and homogenized. RNA was extracted from the *dnIrf6*- or control RNA–injected embryos as described above, without an amplification step. RNA was pooled from a total of approximately 100 embryos of each type; both types were generated on two separate injection days.

Complementary DNA (cDNA) preparation and probe hybridization

For production of the periderm-enriched profile, we synthesized and labeled cDNA once, and hybridized six arrays each with the two classes of probes, using cDNA microarrays in the 12-plex format (Design number 090505_Zv7_EXPR_HX12, Roche NimbleGen, Madison, WI). The periderm-enriched profile was thus generated from a single biological replicate (of about 200 animals) and six technical replicates. On the basis of technical replicates, the false discovery rate-adjusted *P*-values for all genes in the periderm profile were lower than 10^{-8}. For the dnIrf6-inhibited profile, three cDNA synthesis reactions were performed on the pooled RNA, probes were generated from each cDNA preparation, and three arrays (technical replicates) were hybridized with each probe reaction. Differentially expressed genes were selected on the basis of an false discovery rate-adjusted *P*-value cutoff of 0.05, and a fold-change cutoff of 1.7. Because the periderm-enriched and dnIrf6-inhibited profiles were created from embryos at different ages, the periderm-enriched profile was filtered of all genes whose expression at 6 h.p.f. in lacz-injected embryos was not above threshold (defined in Supplementary Methods online) before overlap between the two profiles was evaluated.

Reporter experiments

Construction of reporters to test enhancer function of the human and mouse genomic elements is described in Supplementary Methods online. At 48 h.p.f., 20% (20 of 97 injected embryos) of zebrafish embryos injected with *hGRHL3_peak2:gfp* exhibited patches of 10 or more GFP-positive periderm cells, whereas none of those injected with *hGRHL3_peak1:gfp* (80 injected embryos) or with the pGW_cfosEGFP vector lacking an insert (43 injected embryos) did so.

For both the wild-type Irf6RE and the ΔIrf6RE variant, embryos were injected with 5 nl of plasmid (0.05 $\mu g\,\mu l^{-1}$) plus *tol2* mRNA (25 $ng\,\mu l^{-1}$) at the single-cell stage. Embryos at shield stage (6 h.p.f.) were scored, using a Leica epifluorescence compound microscope (Leica, Wetzlar, Germany), for GFP expression in greater than 10 contiguous periderm cells and persisting until 48 h.p.f. (*mGrhl3intron15:gfp*, 40%, $n=79$ embryos; 0%, *intron15 ΔIrf6RE*, $n=91$ embryos). A 5 μm step z-stack was taken, and the ''auto-blend'' feature of Photoshop CS3 was used to merge it into a single image (Figure 2f). We raised injected founders and identified two that transmitted GFP in the F1 generation. One exhibited high-level GFP expression throughout the periderm, like the transient transgenic embryos (shown in Figure 2h), whereas the other exhibited GFP

expression in facial periderm, in hatching gland cells, and in unidentified oral structures.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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